Peptide Accumulation and Bitterness in Cheddar Cheese Made Using Single-Strain *Lactococcus lactis* Starters with Distinct Proteinase Specificities[1]

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ABSTRACT

This study investigated peptide accumulation and bitterness in reduced- and full-fat Cheddar cheeses that were manufactured with single-strain *Lactococcus lactis* starters that had distinct cell envelope proteinase specificities. Micellar electrokinetic capillary electrophoresis of aqueous cheese extracts detected three large peaks, designated O, P, and Q, that eluted with peptide standards and increased in area during cheese maturation in a pattern that was distinct for each starter. Regression analysis of bitter flavor scores from trained sensory panels and individual O-Q peak areas suggested that peaks P and Q had a negative and positive correlation, respectively, to this defect. Then, HPLC, capillary electrophoresis, peptide sequencing, and mass spectrometry were used to identify five peptides from α_{S1}-casein (CN), one from β-CN, and one from α_{S2}-CN that accumulated in 6-mo-old cheeses. Most of the peptides derived from α_{S1}-CN (f 1–23) accumulated in a manner that corresponded with starter proteinase specificity. All of the peptides identified in the study except α_{S2}-CN (f 1–21) eluted in the O-P-Q region of micellar electrokinetic capillary electropherograms. The α_{S1}-CN (f 1–16), α_{S1}-CN (f 1–17) and β-CN (f 193–209) eluted in peak O, α_{S1}-CN (f 1–13) and α_{S1}-CN (f 1–14) eluted in peak P, and α_{S1}-CN (f 1–9) eluted in peak Q.

(**Key words**: *Lactococcus lactis*, proteinase, cheese proteolysis, bitterness)

Abbreviation key: **A** = absorbance (used with number indicating wavelength), **CEP** = cell envelope proteinase, **FSCE** = free solution capillary electrophoresis, **MECC** = micellar electrokinetic capillary chromatography, **PTA N** = phosphotungstic acid-soluble N.

INTRODUCTION

Proteolysis in Cheddar cheese is an important and complex process that involves endogenous milk enzymes, chymosin, and microbial proteinases and peptidases. Studies of Cheddar cheese ripening suggest that the hydrolysis of intact caseins is almost exclusively catalyzed by chymosin and endogenous milk enzymes. Proteinases and peptidases from *Lactococcus lactis* starters are principally responsible for the production of water-soluble peptides and free amino acids (9). In Cheddar cheese, the concerted action of these enzymes is widely thought to be one of the most important biochemical events during maturation (8). Proteolysis can also lead to bitterness, however, and this defect is observed in reduced- and full-fat Cheddar cheeses (11, 16, 18). Our group is interested in the contribution of starter lactococci to bitterness in situations in which the production of bitter peptides is thought to be a function of lactococcal cell envelope proteinase (**CEP**) specificity and activity (17).

The *L. lactis* CEP is a member of the subtilisin family of serine proteases. Lactococcal CEP exhibit an extremely high degree of amino acid sequence identity, but purified enzymes may differ in their relative affinity for individual caseins and their specificity toward α_{S1}-CN (f 1–23) at near neutral pH (3, 6). Most differences in CEP specificity are due to one or more amino acid substitutions in the enzyme substrate-binding regions (6), and this property has recently been used to classify lactococcal CEP (14). Several research groups (14) have investigated the specificity of purified CEP toward individual caseins, but the specificity of purified CEP differs from that of the native cell-bound form; specificity is also affected by concentrations of acid and salt in cheese (4). Even

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though lactococcal CEP is widely thought to have a central role in cheese proteolysis and flavor development (5), relatively little is known about the specificity of native cell-bound CEP during cheese ripening or the effect of CEP specificity on cheese quality.

This study investigated the influence of lactococcal CEP specificity on peptide accumulation and bitterness in full-fat and 50% reduced-fat Cheddar cheeses. Cheeses were manufactured using single-strain *L. lactis* starters with distinct CEP specificities. Bitterness in the cheeses was evaluated by a trained sensory panel, and a combination of techniques (HPLC, capillary electrophoresis, peptide sequencing, and mass spectrometry) was used to investigate proteolysis.

MATERIALS AND METHODS

Cheese Starter Strains and Growth Conditions

Lactococcus lactis ssp. *cremoris* S1 and *Lactococcus lactis* ssp. *lactis* S3 were acquired from Rhône-Poulenc Dairy Ingredients (Madison, WI). *Lactococcus lactis* ssp. *cremoris* S2 was obtained from the University of Wisconsin-Madison culture collection. Lactococci were propagated at 30°C, stored at 4°C, and maintained by biweekly transfer in M17 broth (21).

L. lactis CEP Classification, Specificity, and Activity

The classification of *L. lactis* ssp. *cremoris* S1, *L. lactis* ssp. *cremoris* S2, and *L. lactis* ssp. *lactis* S3 CEP was performed by DNA sequence analysis of substrate-binding regions, and CEP specificity was investigated by incubation of whole cells with α_{S1}-CN (f 1–23) (4, 6). To characterize CEP substrate-binding regions, DNA fragments that included the subtilisin-like binding region (residues 131, 138, 142, 144, 166, and 177) and the distal binding region (residues 747, 748, and 763) were obtained by polymerase chain reaction in a Perkin-Elmer Applied Biosystems thermocycler (model 480; Foster City, CA) using DNA primers described by Exterkate et al. (6). The amplicons were purified with a Bio-Rad Prep-A-Gene kit (Bio-Rad, Hercules, CA) and then sequenced by fluorescent dideoxy chain termination on a Perkin-Elmer Applied Biosystems automated DNA sequencer (model 373A). Residues in substrate-binding regions were identified by alignment of the deduced amino acid sequences with the *L. lactis* ssp. *cremoris* SK11 group a CEP (6, 22) using GeneWorks version 2.3 software (Intelligenetics, Inc., Mountain View, CA).

Incubations of *L. lactis* whole cells with α_{S1}-CN (f 1–23) were performed using a peptide that had been isolated from chymosin-treated α_{S1}-CN by preparative HPLC as described by Exterkate and Alting (4). Reactions with lactococci and α_{S1}-CN (f 1–23) were also performed as described by those authors except that cells were grown to an absorbance at 600 nm wavelength (A_{600}) = 0.7 in citrated milk that contained 0.5% β-glycerophosphate (24), and 4% NaCl was added to the 25 m*M* Tris-NaH_2PO_4-Na acetate (pH 5.2) reaction buffer to simulate cheese conditions. Samples from each reaction were analyzed by reverse-phase HPLC in a Beckman gradient HPLC system equipped with a 125 dual pump, a 168 diode array detector, and a personal computer-based data system controller (Beckman System Gold version 8.1; Beckman Instruments, Fullerton, CA). The columns were Brownlee Aquapore RP-300 (Perkin Elmer/Applied Biosystems) with 300-Å pores and 7-μm particle size. The column diameter was 2.1 mm for analytical HPLC and 4.6 mm for preparative chromatography; column length was 10 cm. Elutant A was 0.1% (vol/vol) trifluoroacetic acid, and elutant B was 0.085% trifluoroacetic acid in 80% acetonitrile. During each run, the proportion of elutant B in elutant A was increased from 0 to 45% over a 45-min period. Flow rates were 0.2 ml/min for the analytical column and 1 ml/min for the 4.6-mm preparative column. Detection of peptides was performed at 214 nm, and peaks were identified by comparison with the data of Exterkate and Alting (4) and to purified α_{S1}-CN (f 1–9), α_{S1}-CN (f 1–13), α_{S1}-CN (f 1–14), α_{S1}-CN (f 1–16), α_{S1}-CN (f 1–17), and α_{S1}-CN (f 1–23) peptide standards.

Lactococcal CEP activity was measured using the EnzChek protease kit (Molecular Probes, Eugene, OR) with fluorescent-labeled casein as described by the manufacturer. Lactococci were grown to A_{600} = 0.7 in citrated milk that contained 0.5% β-glycerophosphate; the cells were centrifuged at 6000 × *g* for 10 min, washed with one volume of ice cold 0.85% saline and 20 m*M* $CaCl_2$, and then suspended in 0.4 ml of wash solution. Samples (100 μl) of each cell suspension were then transferred to a test tube that contained 1 ml of substrate and 0.9 ml of 10 m*M* Tris·HCl (pH 7.8). The reactions were incubated for 24 h at 30°C in the dark, and fluorescence was measured (excitation, 480 nm; emission, 520 nm) in a scanning fluorometer (model RF1501; Shimadzu, Columbia, MD). The number of colony-forming units per milliliter in each reaction tube was determined by plate count on Elliker's agar before the assay, and all reactions were performed in duplicate with sterile

spent culture media included as a negative control. Lactococcal CEP activity per cell was then calculated by dividing the mean relative fluorescence units by the number of colony-forming units per milliliter.

Cheddar Cheese Manufacture

Cheddar cheese was manufactured on two separate occasions from 250-kg lots of milk at the University of Wisconsin-Madison. Cheeses made during yr 1 of the study were used to investigate the effect of different cultures on 50% reduced-fat Cheddar cheese; cheeses manufactured during yr 2 were used to compare the effects of those cultures in reduced-fat versus full-fat Cheddar cheese.

Vats of 50% reduced-fat Cheddar cheese were manufactured from raw, whole milk skimmed to 1.3% fat and pasteurized at 73.3°C for 16 s. *Lactococcus lactis* ssp. *cremoris* S1, *L. lactis* ssp. *cremoris* S2, and *L. lactis* ssp. *lactis* S3 were grown separately at 30°C for 12 to 14 h in skim milk that had been steamed for 45 min. Duplicate vats were inoculated with 1.25% (wt/wt) of S1, 2.0% of S2, or 0.5% of S3 to obtain a uniform rate of acid production in each cheese. Some vats of yr 1 reduced-fat cheese were also inoculated with flavor adjunct bacteria at 10^4 to 10^5/ml. The identity of those adjuncts and their influence on cheese flavor development were discussed by Weimer et al. (23).

Fifteen minutes after starter inoculation, 49 ml of calcium chloride (Rhône-Poulenc, Madison, WI) and 19 ml of double-strength fermentation-produced chymosin (Pfizer, Inc., Milwaukee, WI) were added. The coagulum was cut at a milk pH of 6.5 with 0.95-cm knives and then allowed to heal for 5 min. After 10 min of gentle agitation, the temperature of the curd and whey slurry was raised from 32 to 37.8°C over 25 min; then the whey was slowly drained. Cheese slabs were cheddared and then milled when the curd reached pH 5.95. Fifteen minutes after milling, the curd was salted in three additions, 5 min apart, with 0.275% (wt/wt) flake salt (calculated from the original milk weight). The salted curd was packed into 9-kg rectangular stainless steel hoops, pressed for 4 h at ambient temperature, vacuum-packaged, and stored at 7°C for ripening.

Full-fat Cheddar cheese manufactured in the 2nd yr of the study was produced from pasteurized whole milk essentially as described except that duplicate vats were inoculated with 1.75% (wt/wt) of S1, 2.0% of S2, or 1.0% of S3 to obtain a uniform rate of acid production, the coagulum was cut with 0.63-cm knives, the temperature of the curd and whey slurry was raised from 32 to 38.3°C over 30 min, the cheese slabs were milled when the curd reached pH 5.55, and the curd was salted with 0.3% (wt/wt) flake salt.

Compositional Analysis

Fat, moisture, and salt contents and pH of cheese were determined on d 1 as described previously (23). Cheese pH was measured again at 2 mo. Samples were also collected once per month for the enumeration of starter and nonstarter colony-forming units. Cheese for microbiological sampling was homogenized in 2% citrate at 45°C, and then total bacterial counts were collected by pour plating appropriate dilutions in Elliker's agar (Difco). Nonstarter (or adjunct) lactobacilli were also enumerated using Rogosa SL agar (Difco). Plates were incubated anaerobically for 2 d at 30°C (Elliker's) or 37°C (Rogosa), and numbers of starter colony-forming units were determined by subtracting the *Lactobacillus* count from the total bacterial count. The production of free amino acids and very small peptides (<600 Da) in cheese was also followed each month by determination of 5% phosphotungstic acid-soluble N (**PTA N**) (12).

Sensory Evaluation of Bitterness

The sensory attributes of cheese samples were evaluated at the University of Wisconsin-Madison by 6 to 10 experienced judges who were sensitive to the bitterness attribute. Experimental cheeses were coded and presented in a randomized order to panelists, to be judged for several attributes, including bitter flavor intensity (where 1 = none, 3 = slight, 5 = definite, and 7 = pronounced). Judges initially met as a group to set consensus ratings for all flavor categories using a full-fat control cheese. Consensus scores were marked on ballot sheets, and judges individually evaluated sample cheeses by category scaling against the reference (control) cheese. Statistical analysis of trained sensory data used a completely randomized split plot with repeated measures design as described by Weimer et al. (23).

Primary and Secondary Proteolysis

Cheese proteolysis was monitored by a combination of capillary electrophoresis methods and HPLC. Capillary electrophoresis was performed with a PACE 2100 automated system (Beckman Instruments, Inc.) equipped with an untreated silica capillary (75 μm i.d. $\times$ 57 cm) and System Gold software (version 7.11). New capillaries were washed with 1 *M* NaOH, equilibrated with run buffer until the baseline stabi-

lized (typically overnight), and then dedicated to the equilibration buffer. Voltages were kept within the linear range of an Ohm's law plot of voltage and current at 25°C and were ramped to the desired value in 1 min. Sample detection was achieved at 200 nm with the detector range at 0.02 AUFS (absorbance units full scale) and a data collection rate of 2 Hz. The polarity was set with the positive pole at the capillary inlet.

The hydrolysis of α_{S1}- and β-CN in ripening cheese was followed by free solution capillary electrophoresis (**FSCE**) at 25°C using 100 m*M* sodium phosphate and 4 *M* urea (final pH 3.35) buffer (prepared from a 200 m*M* sodium phosphate stock solution [pH 2.75]). New capillaries for phosphate-urea FSCE were conditioned in buffer for at least 24 h prior to use and stored in buffer when not in use. Samples were prepared by dissolving 0.5 g of ground cheese in 10 ml of a freshly prepared solution of 10 *M* urea and 50 m*M* phosphoric acid. The sample was stirred for approximately 1 h until cheese pieces were dissolved and centrifuged for 10 min at 16,000 × *g* to remove the lipid fraction. The aqueous layer was collected, filtered through a 0.2-μm low protein-binding filter (Gelman Sciences, Ann Arbor, MI), and then stored at –20°C until needed, but never more than 1 wk. Immediately prior to analysis, samples were thawed, and appropriate dilutions were prepared in 4 *M* urea and 12.5 m*M* phosphoric acid (pH 4.0) sample buffer. The FSCE was then performed as described by Strickland et al. (20), except that the washing steps between runs were modified as follows: a 2-min rinse with 2× sample buffer, followed by a 2-min rinse with 0.2 *M* sodium phosphate, pH 2.75, and then a 4-min rinse with 100 m*M* sodium phosphate and 4 *M* urea.

Secondary proteolysis in the cheeses was studied by a combination of micellar electrokinetic capillary chromatography (**MECC**), FSCE, and HPLC. Aqueous Cheddar cheese extracts that had been prepared as described by Strickland et al. (20), except that double deionized water was used instead of 100 m*M* phosphate for extractions in yr 2 of the study. The MECC was performed using 100 m*M* sodium borate (pH 8.5) with 40 m*M* SDS as described by Strickland et al. (20). The FSCE in 100 m*M* sodium phosphate (pH 2.74) buffer was performed as described by Strickland and Strickland (19). Cheese extracts for acid phosphate FSCE were size-fractionated using 3000 molecular mass cutoff membranes (Amicon, Beverly, MA) and washed several times with distilled water. The retentate was collected and stored at –20°C until needed. Samples were run for 30 min at 15 kV followed by a 7-min low pressure rinse. Prior to use, the capillary was conditioned overnight with 500 m*M* sodium phosphate (pH 2.50). Before each run, a 1-min high pressure rinse with 100 m*M* sodium phosphate (pH 2.74) was performed. Peptide solutions (ca. 20 μg/ml) in 25 m*M* sodium phosphate (pH 2.74) were injected with 1 to 5-s injections. At the end of a run, two 1-min forward high pressure rinses were performed with 0.1 *M* HCl and 500 m*M* sodium phosphate (pH 2.50).

Cheese samples for reverse-phase HPLC were prepared as described for acid phosphate FSCE. Separations were performed with a Beckman gradient HPLC system as described for the study of CEP specificity.

Peptide Characterization

The peptides that were isolated from sample cheeses were identified by amino-terminal sequencing and mass determination. Samples for each analysis were purified by HPLC as described and then were lyophilized in a benchtop freeze-dryer (model 5L; VirTis, Gardiner, NY). Amino-terminal sequencing was performed by Edman degradation in a protein sequencer (model 477B; Applied Biosystems). Peptides for mass spectrometry were dissolved in 0.1% trifluoroacetic acid and analyzed by matrix-assisted laser desorption ionization time of flight on a TofSpec (Micromass, Beverly, MA) mass spectrometer with external mass calibration. The spectrometer was set at 20 kV in the linear mode with an N_2 laser (337 nm), and the matrix was α-cyano-4-hydroxy-cinnamic acid. Finally, the mean hydrophobicity and molecular mass of each peptide were calculated (PeptID 93 software; University of Minnesota, St. Paul).

RESULTS

Lactococcal CEP Classification and Specificity

Deduced amino acid sequences for the *L. lactis* ssp. *cremoris* S1 CEP substrate-binding regions showed that this enzyme belonged to CEP group e (Table 1) (14). *Lactococcus lactis* ssp. *cremoris* S2 was originally designated as strain SK11 (23), but SK11 has a group a CEP (14), and DNA sequence analysis of S2 CEP substrate-binding regions showed it was a group b enzyme (Table 1). As is also shown in Table 1, DNA sequence analysis of the S3 CEP substrate-binding regions revealed this enzyme had a distal binding region that was identical to that found in CEP groups f and g, but the subtilisin-like binding region was unlike any previously described CEP group.

TABLE 1. Classification of *Lactococcus lactis* ssp. *cremoris* S1, *L. lactis* ssp. *cremoris* S2, and *Lactococcus lactis* ssp. *lactis* S3 cell envelope proteinase (CEP) from deduced amino acid sequences for substrate binding regions.[1]

Strain	Residues in substrate binding regions									CEP Classification
	131	138	142	144	166	177	747	748	763	
S1	Thr	Thr	Ser	Leu	Asp	Leu	Leu	Thr	Asn	Group e
S2	Thr	Thr	Ala	Leu	Asp	Leu	Arg	Lys	Asn	Group b
S3	Thr	Thr	Ala	Leu	Gly	Ile	Leu	Thr	His	Group h[2]

[1]Determined by comparison to the data of Exterkate et al. (8).

[2]A new group designation was assigned because CEP with identical substrate binding regions have not been previously described.

Differences in the specificity of S1, S2, and S3 CEP were evident in peptide profiles obtained after brief (15 to 30 min) and prolonged (120 to 180 min) incubations under cheese-like conditions (pH 5.2, 4% NaCl) of whole cells with α_{S1}-CN (f 1–23) (4). Brief incubations with S2 indicated the group b CEP of this bacterium had a strong affinity for the Leu_{16}-Asn_{17} and Asn_{17}-Glu_{18} bonds of α_{S1}-CN (f 1–23), but α_{S1}-CN (f 1–13) and some α_{S1}-CN (f 1–9) were also detected (Figure 1A). Similar experiments with S1 suggested that the group e CEP of this bacterium had a specificity toward α_{S1}-CN (f 1–23) that resembled strain HP group g CEP (6, 14). Each of these enzymes preferentially hydrolyzed α_{S1}-CN (f 1–23) at the Gln_{13}-Glu_{14} position, but α_{S1}-CN (f 1–16) and α_{S1}-CN (f 1–9) were also formed (Figure 1, B and D). Thirty-minute incubations with S3 indicated its CEP had the greatest affinity for the Leu_{16}-Asn_{17} and Gln_{13}-Glu_{14} bonds of α_{S1}-CN (f 1–23), and cleavage at Gln_9-Gly_{10} as well (Figure 1C).

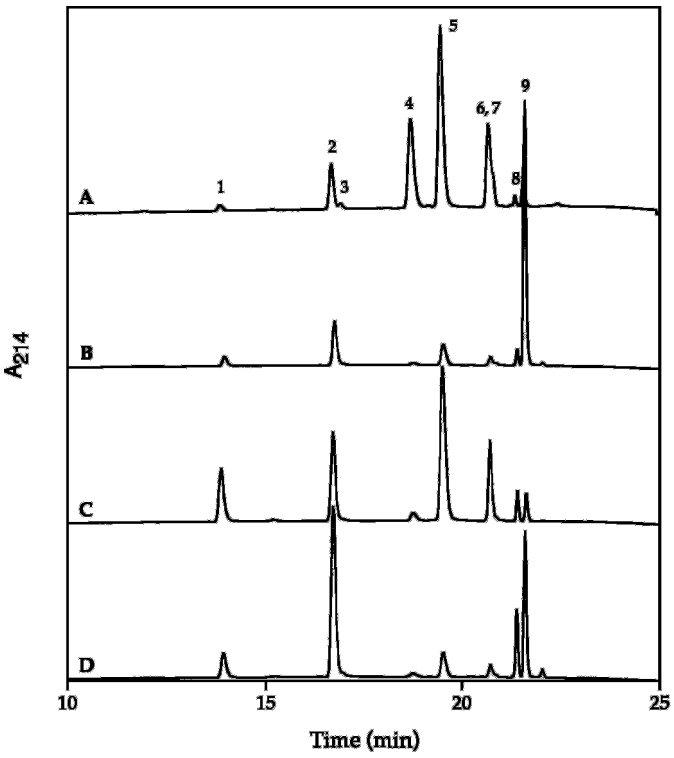

Figure 1. Reversed-phase HPLC of the products of α_{S1}-CN (f 1–23) after incubation with whole cells of *Lactococcus lactis* ssp. *cremoris* S2 (A), *L. lactis* ssp. *cremoris* S1 (B), *Lactococcus lactis* ssp. *lactis* S3 (C), or *L. lactis* ssp. *cremoris* HP (D). Incubations were performed at 30°C for 15 (S2) or 30 min (S1, S3, and HP) in 25 m*M* Tris-NaH_2PO_4-Na acetate (pH 5.2) (4) with 4% NaCl. Peptides identified in the chromatograms include peak 1, α_{S1}-CN (f 1–9); peak 2, α_{S1}-CN (f 1–13); peak 3, α_{S1}-CN (f 1–14); peak 4, α_{S1}-CN (f 1–17); peak 5, α_{S1}-CN (f 1–16); peak 6, α_{S1}-CN (f 17–23); peak 7, α_{S1}-CN (f 18–23); peak 8, α_{S1}-CN (f 14–23); and peak 9, α_{S1}-CN (f 1–23). A_{214} = Absorbance at 214 nm wavelength.

Prolonged incubations of S1 or S2 with α_{S1}-CN (f 1–23) showed little change in the peptide patterns for these strains (Figure 2, A and B), but similar experiments with S3 gave a new pattern that was apparently due to efficient secondary conversion of α_{S1}-CN (f 1–16) to α_{S1}-CN (f 1–9) by the S3 CEP (Figures 1C and 2C).

Cheese Composition

Percentages of fat, moisture, and salt in moisture contents were very similar among reduced-fat (13.5 ± 0.2, 48.2 ± 0.9, and 3.4 ± 0.2, respectively) or full-fat (31.7 ± 0.5, 38.4 ± 0.5, and 4.2 ± 0.3) Cheddar cheeses that had been manufactured with S1, S2, or S3 single-strain starters. Cheese pH at 2 mo was also similar (range = pH 5.0 to 5.2).

Reduced-fat Cheddar cheese made in yr 1 with S1 or S2 contained approximately 10^8 cfu/g of starter at pressing, and the numbers of these bacteria declined about 1 log during the 1st mo of ripening (Table 2). In contrast, yr 1 cheese that was made with S3 contained well over 10^9/g of starter at d 1, and starter numbers remained high even after 1 mo of ripening. As shown in Table 2, the trend in starter numbers for reduced-fat cheese made with S2 in yr 2 was similar to that of cheese made in yr 1, but numbers of starter bacteria in all other yr 2 cheeses did not show any decrease during the 1st mo of ripening.

Incubations with whole cells and fluorescent casein indicated that strains S1, S2, and S3 had relative CEP activities of 15.4, 10.1, and 3.7 RFU/cfu, respectively. If one assumes that the differences in relative CEP activity for strains grown in citrated milk are essentially conserved during growth in regular milk, initial starter numbers for each cheese (Table 2) suggest that total CEP activity probably was at least 10-fold higher in reduced-fat cheeses made in yr 1 with S3 versus that made with S1 or S2. A similar disparity was calculated for total CEP activity in reduced-fat and full-fat cheeses made in yr 2 with S3 versus S1, although total CEP activity in yr 2 cheeses made with S2 was approximately 5-fold less than that in S3 cheeses.

The differences that were noted in total CEP activity were not reflected in PTA N concentrations. The PTA N in reduced-fat cheeses made in both years of the study was highest for cheese made with S2, followed by S1, and then S3. The PTA N concentrations in full-fat Cheddar were highest in S1 cheese, followed by S3 and then S2 cheese (data not included).

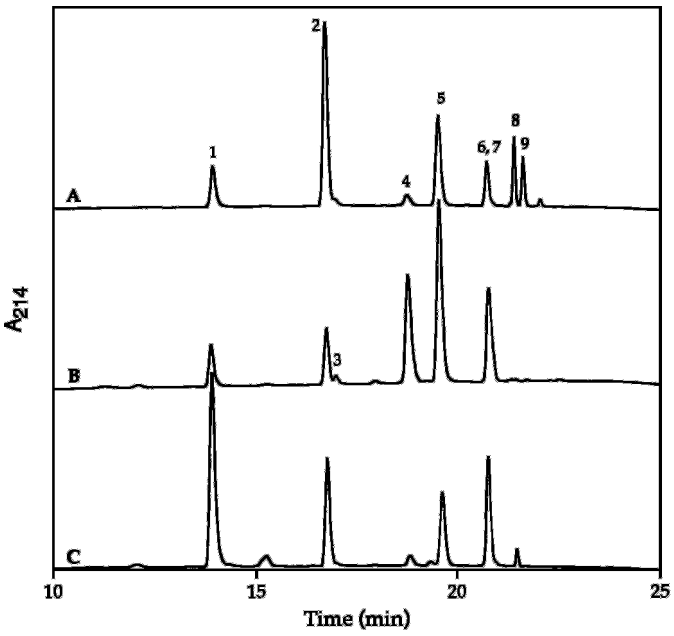

Figure 2. Reversed-phase HPLC of the products of α_{S1}-CN (f 1–23) after prolonged incubation with whole cells of *Lactococcus lactis* ssp. *cremoris* S1 (A), *L. lactis* ssp. *cremoris* S2 (B), or *Lactococcus lactis* ssp. *lactis* S3 (C). Incubations were performed at 30°C for 180 (S1) or 120 min (S2, S3) in 25 m*M* Tris-NaH_2PO_4-Na acetate (pH 5.2) (4) with 4% NaCl. Peptides identified in the chromatograms include peak 1, α_{S1}-CN (f 1–9); peak 2, α_{S1}-CN (f 1–13); peak 3, α_{S1}-CN (f 1–14); peak 4, α_{S1}-CN (f 1–17); peak 5, α_{S1}-CN (f 1–16); peak 6, α_{S1}-CN (f 17–23); peak 7, α_{S1}-CN (f 18–23); peak 8, α_{S1}-CN (f 14–23); and peak 9, α_{S1}-CN (f 1–23). A_{214} = Absorbance at 214 nm wavelength.

Primary Proteolysis in Reduced-Fat Cheddar Cheese

Phosphate-urea FSCE was used to investigate primary proteolysis in reduced-fat Cheddar cheese made in this study. In full-fat Cheddar cheese, hydrolysis of intact α_{S1}- and β-CN during ripening occurs primarily through the action of residual chymosin and native plasmin, respectively (10). Coinjection studies with purified α_{S1}- and β-CN (20), purified α_{S1}-CN (f 1–9) and α_{S1}-CN (f 1–13), and partially purified α_{S1}-CN (f 24–199) showed that, at press, reduced-fat Cheddar cheeses made with S1 or S3 single-strain starters contained three large peaks, which corresponded to intact α_{S1}- and β-CN, as well as a small peak, which comigrated with the chymosin-derived peptide α_{S1}-CN (f 24–199) (Figure 3). After 2 mo of ripening, concentrations of intact β-CN had decreased only slightly, but α_{S1}-CN was almost completely hydrolyzed, and the peak for α_{S1}-CN (f 24–199) had become several times larger. Those changes indicated that residual chymosin had a more active role than plasmin in primary proteolysis during the first 2 mo of ripening. Two-month-old cheese also contained two small peaks that comigrated with α_{S1}-CN (f 1–9) and α_{S1}-CN (f 1–13). Those peptides are produced from the chymosin-derived peptide α_{S1}-CN (f 1–23) by starter bacteria (5). By 4 mo, intact α_{S1}-CN had essentially disappeared, and β-CN peaks were approximately one-half of the area that was noted at press. Peaks for α_{S1}-CN (f 24–199), α_{S1}-CN (f 1–9), and α_{S1}-CN (f 1–13) showed only minor changes from 2 to 4 mo, which suggested that the production and conversion of these peptides was approaching equilibrium.

TABLE 2. Numbers of viable *Lactococcus lactis* ssp. *cremoris* S1, *L. lactis* ssp. *cremoris* S2, or *Lactococcus lactis* ssp. *lactis* S3 starter bacteria in reduced-fat and full-fat Cheddar cheeses.

	Single-strain starter		
	S1	S2	S3
	(cfu/g)		
Reduced-fat cheese			
yr 1			
d 1 (press)	1×10^8	2×10^8	7×10^9
>1 mo	7×10^6	2×10^7	3×10^9
yr 2			
d 1 (press)	6×10^7	3×10^8	3×10^9
>1 mo	6×10^8	7×10^7	1×10^{10}
Full-fat cheese			
yr 2			
d 1 (press)	4×10^8	2×10^9	3×10^{10}
>1 mo	1×10^9	2×10^9	6×10^{10}

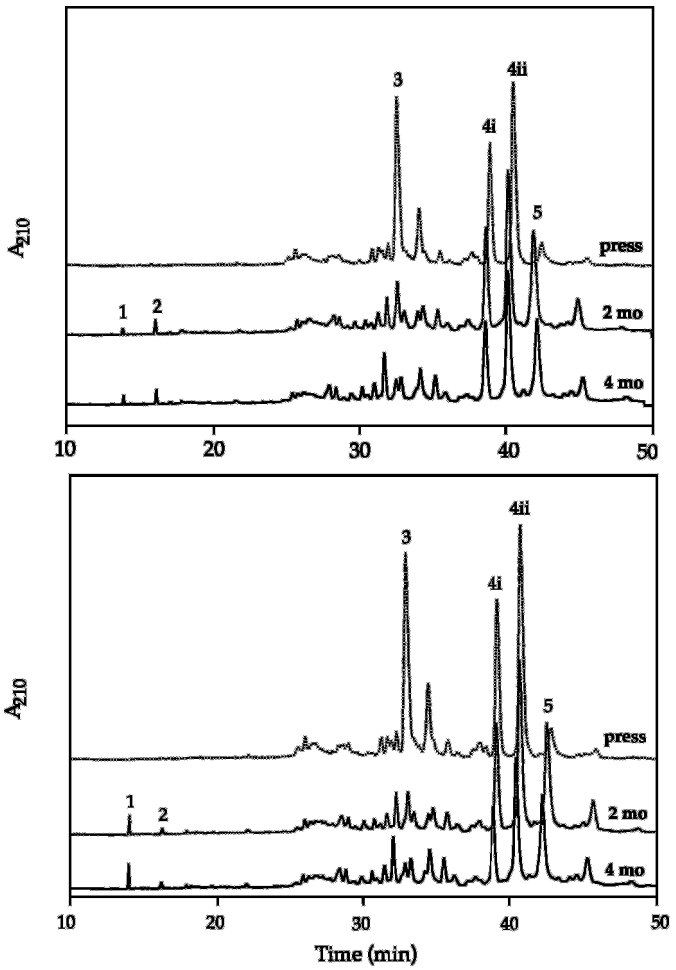

Figure 3. Free solution capillary electrophoresis in phosphate-urea of reduced-fat Cheddar cheese made in yr 2 with *Lactococcus lactis* ssp. *cremoris* S1 (upper panel) or *Lactococcus lactis* ssp. *lactis* S3 (lower panel) single-strain starters. Peaks identified by coinjection with purified standard peptides or caseins include peak 1, α_{S1}-CN (f 1–9); peak 2, α_{S1}-CN (f 1–13); peak 3, α_{S1}-CN; peak 4i, β-CN A^1; peak 4ii, β-CN A^2; and peak 5, α_{S1}-CN (f 24–199). A_{210} = Absorbance at 210 nm wavelength.

Bitter Flavor Intensity

Evaluation by the trained sensory panel showed that yr 1 cheeses, made with S1 or S2, did not develop bitter flavors during 6 mo of ripening (maximum bitter flavor intensity score = 1.3), but cheese made with S3 was slightly bitter by 2 mo (bitter flavor intensity = 2.0 ± 0.1) and definitely bitter after 4 and 6 mo of ripening (bitter flavor intensity = 4.4 ± 0.6 and 5.4 ± 0.8, respectively). The ANOVA showed that starter choice was the only factor that significantly influenced bitterness in Cheddar cheese ($\alpha = 0.05$; $P < 0.0005$), and that effect was clearly due to S3. Cheeses made with S3 single-strain starters in the 2nd yr of the study were also bitter. Six-month-old reduced- and full-fat Cheddar cheeses made with S3 each received mean scores for intensity of bitter flavor of 4.5. In comparison, 6-mo-old reduced-fat cheese made with S1 or S2 received bitter flavor scores of 2.7 and 1.7, respectively; full-fat cheese made with those starters had bitter flavor scores of 1.5 and 2.1.

MECC Profile and Bitterness

The MECC electropherograms of aqueous cheese fractions are complex, but characteristically contain three prominent peaks, which elute just before the micelle marker (20). As shown in Figure 4, the total peak area for these peaks, designated O, P, and Q, increased during maturation in a pattern that was characteristic for each starter culture. Correlation analysis between individual O-P-Q peak areas and bitter flavor scores from 16 cheeses made with each starter in yr 1 showed no obvious correlation to peak O, but peaks P and Q gave a negative and positive correlation with bitterness, respectively, that increased as ripening time increased. The correlation coefficient between peak P area and bitterness at 2, 4, and 6 mo in reduced-fat Cheddar made in yr 1 was –0.58, –0.73, and –0.81, respectively; for peak Q and bitterness, r = 0.66, 0.79, and 0.83. Similar results were obtained for Cheddar cheeses made in duplicate with each starter in the 2nd yr of the study. For peaks P and Q with bitterness in 6-mo-old Cheddar made in yr 2, r = –0.80 and 0.83, respectively, for reduced-fat cheese, and r = –0.88 and 0.77 for full-fat Cheddar.

Peptide Identification and Relative Abundance in Cheese

As shown in Figure 5, aqueous extracts of cheese that were made from each starter also had characteristic HPLC and acid phosphate FSCE profiles. The HPLC peaks A through G (Figure 5) were individually collected from 6-mo-old reduced-fat cheese and then were analyzed by MECC and acid phosphate FSCE to evaluate fraction purity and to ascertain electrophoretic mobility on capillary electropherograms. Analysis of the amino-terminal peptide sequence and mass spectrometry of HPLC fractions identified five peptides from α_{S1}-CN, one peptide from α_{S2}-CN, and one from β-CN, which accumulated in reduced-fat Cheddar cheese (Table 3). As shown in Figure 5 and Table 4, levels of individual peptides in reduced- or full-fat Cheddar cheese differed in a manner corresponding with the starter bacterium that was used to produce each cheese. Except for the α_{S2}-CN fragment, all of the peptides that were identified

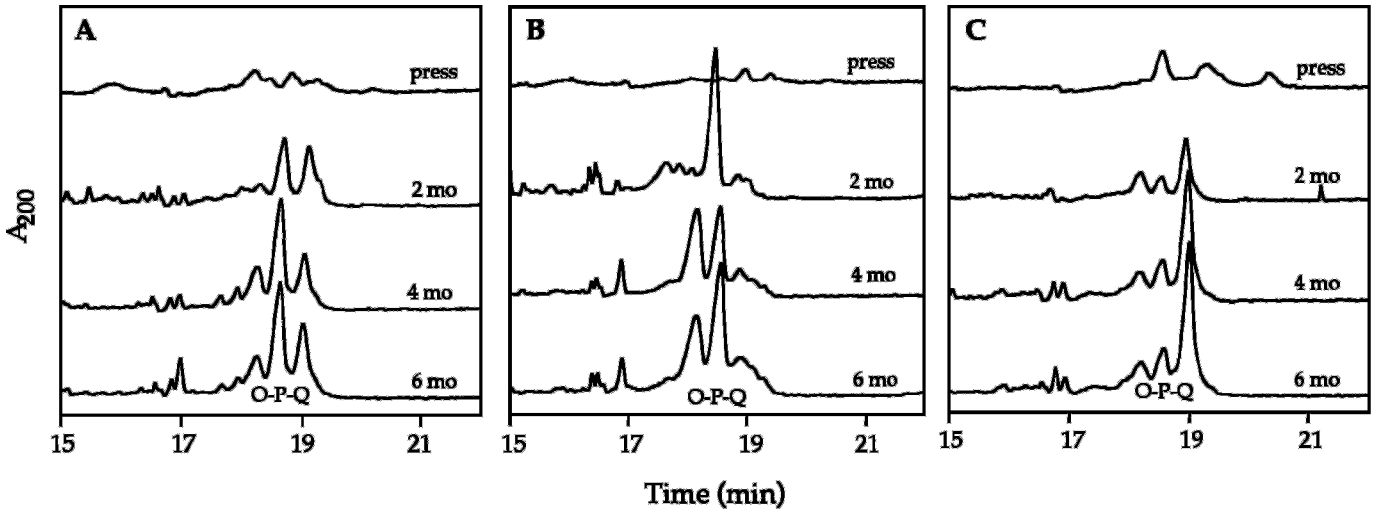

Figure 4. The 15- to 21-min region of micellar electrokinetic capillary chromatograms obtained from the aqueous fraction of 6-mo-old reduced-fat cheese produced in yr 1 using *Lactococcus lactis* ssp. *cremoris* S1 (A), *L. lactis* ssp. *cremoris* S2 (B), or *Lactococcus lactis* ssp. *lactis* S3 (C) single-strain starters. The letters O, P, and Q identify the three large peaks. A_{200} = Absorbance at 200 nm wavelength.

in this study were relatively hydrophobic molecules (Q >1300) (17), which eluted in the MECC O-P-Q peak region.

DISCUSSION

This study investigated peptide accumulation and bitterness in reduced- and full-fat Cheddar cheeses that were manufactured with single-strain *L. lactis* starters that produce distinct CEP. Two of the starters, S1 and S2, are used commercially in the US for Cheddar cheese manufacture; the third, S3, is known to produce bitter flavors in milk consistently (J. Kondo, 1993, personal communication). As shown in Table 1, S1 and S2 produced group e and group b CEP, respectively. Characterization of the S3 CEP showed this enzyme had substrate binding regions that were unlike any previously described CEP group (Table 1), and its specificity toward α_{S1}-CN (f 1–23) under cheese-like conditions was also unique (Figures 1 and 2). These data showed that the S3 enzyme represented a new CEP group, which was designated in this study as group h.

Cheddar cheese manufactured with single-strain starters S1, S2, or S3 had similar moisture, fat, and salt in moisture contents and pH values. Phosphate-urea FSCE electropherograms of ripening reduced-fat cheese (Figure 3) suggested that, like full-fat Ched-

TABLE 3. Characteristics of peptides isolated in this study from Cheddar cheese.

	Elution peak or time				Mass[4]	
Peptide[1]	HPLC	MECC[2]	FSCE	Hydrophobicity[3]	Actual	Calculated
α_{S1}-CN (f 1–9)	A	Q	I	1422	1141	1143
α_{S1}-CN (f 1–13)	B	P	II	1363	1537	1538
α_{S1}-CN (f 1–14)	C	P	III	1305	1664	1666
α_{S1}-CN (f 1–16)	F	O	IV	1399	1874	1878
α_{S1}-CN (f 1–17)	E	O	V	1316	1988	1993
α_{S2}-CN (f 1–21)	D	~12 min	>30 min	880	2427[5]	2425
β-CN (f 193–209)	G	O	>30 min	1796	1881	1881

[1]Identified by amino terminal sequence analysis and mass spectrometry.

[2]MECC = Micellar electrokinetic capillary chromatography; FSCE = free solution capillary electrophoresis.

[3]Peptide average hydrophobicity as determined by PeptID software (University of Minnesota, St. Paul).

[4]Peptide mass as determined by mass spectrometry and as calculated from amino acid composition.

[5]Mass spectrometry performed after peptide dephosphorylation with calf intestine alkaline phosphatase (1).

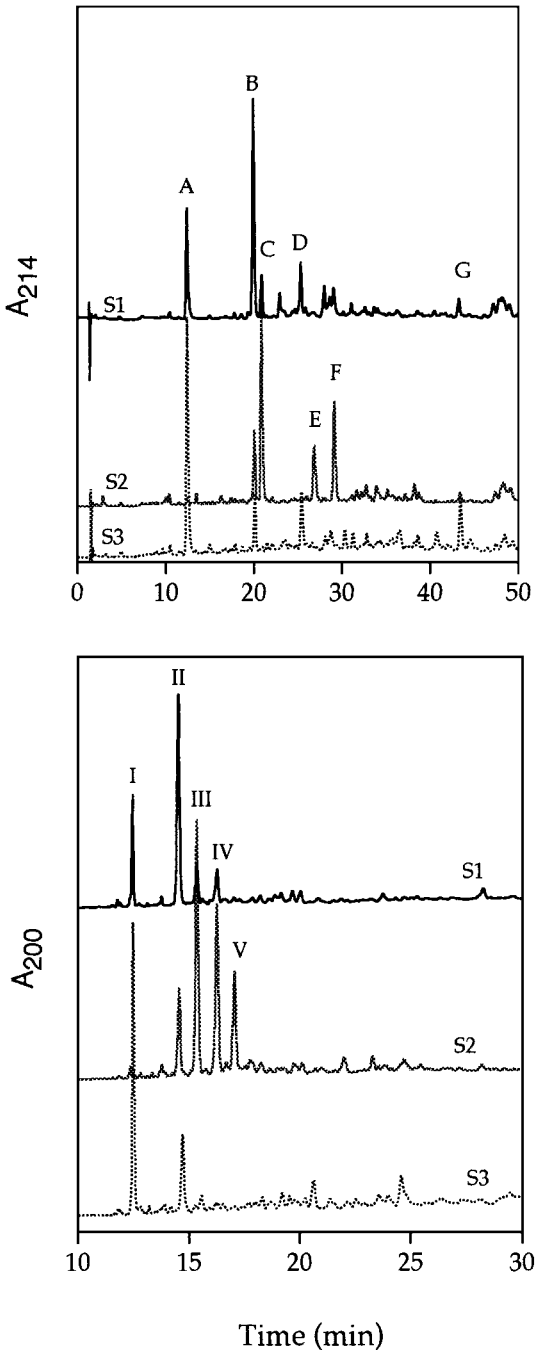

Figure 5. Reversed-phase HPLC chromatograms (upper panel) and acid phosphate-free solution capillary electropherograms (lower panel) of size-fractionated aqueous extracts of 6-mo-old reduced-fat Cheddar cheese manufactured in yr 1 with *Lactococcus lactis* ssp. *cremoris* S1, *L. lactis* ssp. *cremoris* S2, or *Lactococcus lactis* ssp. *lactis* S3 single-strain starters. Peptides identified in the chromatogram include peak A, α_{S1}-CN (f 1–9); peak B, α_{S1}-CN (f 1–13); peak C, α_{S1}-CN (f 1–14); peak D, α_{S2}-CN (f 1–21); peak E, α_{S1}-CN (f 1–17); peak F, α_{S1}-CN (f 1–16); and peak G, β-CN (f 193–209). Peptides in the electropherogram were identified as peak I, α_{S1}-CN (f 1–9); peak II, α_{S1}-CN (f 1–13); peak III, α_{S1}-CN (f 1–14); peak IV, α_{S1}-CN (f 1–16); and peak V, α_{S1}-CN (f 1–17). A_{200} or A_{214} = Absorbance at 200 or 214 nm wavelength.

dar (10), primary proteolysis in this variety was essentially a consequence of chymosin and plasmin activity. The MECC electropherograms of reduced- and full-fat cheeses all contained three large terminal peaks, the total area of which increased during maturation in a pattern that was unique for each starter culture (Figure 4). Because small hydrophobic peptide standards also eluted in this region (20), we suspected that these peaks contained peptides and that differences in MECC patterns for this region reflected differences in the activity and specificity of starter proteolytic enzymes. Experiments to investigate this hypothesis led to the identification of one peptide from α_{S2}-CN, five peptides from α_{S1}-CN, and one peptide from β-CN that accumulated in ripening Cheddar cheese.

To our knowledge, α_{S2}-CN (f 1–21) has not been previously isolated from cheese, but plasmin is known to cleave α_{S2}-CN at the Lys_{21}-Gln_{22} position (9). The α_{S2}-CN (f 1–21) peptide was detected in all cheeses, but concentrations were characteristically low in cheeses made with S2 (Figure 5 and Table 4). This observation indicated that the group b CEP or intracellular peptidases from S2, or both, served to limit accumulation of α_{S2}-CN (f 1–21) in S2 cheese.

The peptides α_{S1}-CN (f 1–9), α_{S1}-CN (f 1–13), and α_{S1}-CN (f 1–14) that were identified in this study were also found by Kaminogawa et al. (13) to accumulate in Gouda cheese. Those authors (13) also showed these peptides were produced from purified α_{S1}-CN (f 1–23) by proteases in a cell-free extract of *L. lactis* ssp. *cremoris* H61. That work was followed by a study by Exterkate and Alting (5), who showed that peptides derived from α_{S1}-CN (f 1–23) in 2-wk-old Gouda cheese reflected the in vitro CEP specificity of the starter that had been used to produce the cheese; those researchers (5) concluded that CEP specificity during early proteolysis might be important for the course of gross proteolysis during the later stages of ripening. Comparisons between the relative abundance of peptides derived from α_{S1}-CN (f 1–23) in 6-mo-old Cheddar cheese that was manufactured in this study with S1, S2, or S3 single-strain starters showed that starter CEP specificity was heavily reflected in the pool of peptides that accumulated in ripened cheese. Peptides derived from α_{S1}-CN (f 1–23) accumulated in 6-mo-old Cheddar cheeses made with S1, S2, or S3 (Figure 5 and Table 4) in a manner that was consistent with results from in vitro studies of CEP specificity that were performed under cheese-like conditions (Figure 2). One exception to this observation was α_{S1}-CN (f 1–14), which accumulated in some cheeses even though it

TABLE 4. Relative abundance of individual peptides in size-fractionated aqueous extracts of 6-mo-old reduced-fat and full-fat Cheddar cheeses manufactured in yr 2 with *Lactococcus lactis* ssp. *cremoris* S1, *L. lactis* ssp. *cremoris* S2, or *Lactococcus lactis* ssp. *lactis* S3 single-strain starters.[1]

	Reduced-fat cheese			Full-fat		
Peptide	S1	S2	S3	S1	S2	S3
α_{S1}-CN (f 1–9)	9.7	1.6	18.1	10.8	ND[2]	20.4
α_{S1}-CN (f 1–13)	11.7	10.2	6.6	20.7	4.2	5.0
α_{S1}-CN (f 1–14)	2.5	22.8	3.0	4.6	8.4	ND[2]
α_{S1}-CN (f 1–16)	1.6	1.0	<1.0	ND[2]	11.0	ND[2]
α_{S1}-CN (f 1–17)	ND[2]	2.8	ND[2]	ND[2]	4.1	ND[2]
α_{S2}-CN (f 1–21)	5.7	<1.0	7.0	5.7	3.3	10.2
β-CN (f 193–209)	3.0	ND[2]	2.3	2.0	4.7	5.6

[1]Numbers depict the percentage of the total peak area on respective HPLC chromatograms represented by the peak area for each peptide. Peak areas for each peptide were obtained from the HPLC chromatogram of a representative cheese made with each starter and divided by the total peak area for that chromatogram.

[2]Not detected.

was not a major product of S1, S2, or S3 CEP (Table 4; Figures 1 and 2). The appearance of α_{S1}-CN (f 1–14) in cheese can be explained by the work of Baankreis et al. (2), which demonstrated that α_{S1}-CN (f 1–23), α_{S1}-CN (f 1–16), and α_{S1}-CN (f 1–17) were hydrolyzed to α_{S1}-CN (f 1–13) and α_{S1}-CN (f 1–14) by the lactococcal neutral oligoendopeptidase, PepO. The activity of PepO, an intracellular enzyme, in the cheese matrix arose as a direct consequence of starter lysis (2, 5).

A contribution by PepO to peptide conversion in Cheddar cheese was evident from comparisons between the peptide content of reduced-fat versus full-fat Cheddar cheese made with *L. lactis* ssp. *cremoris* S2. As shown in Table 2, numbers of viable starter bacteria in full-fat S2 cheese remained constant during the 1st mo of ripening, so starter autolysis (and thus PepO activity) was probably less significant in those cheeses than in S2 reduced-fat cheeses for which starter numbers had declined by approximately 1 log during the same period. As would be expected in a cheese with lower PepO activity, full-fat S2 cheese contained much higher concentrations of α_{S1}-CN (f 1–16) and substantially lower concentrations of α_{S1}-CN (f 1–13) and α_{S1}-CN (f 1–14) than reduced fat cheese made with S2 (Table 4). Full-fat S2 cheese also contained higher levels of β-CN (f 193–209), which can be efficiently hydrolyzed by PepO but not by CEP (2, 7).

The β-CN (f 193–209) peptide is a product of chymosin (9) and has been associated with bitterness in cheese (17). Although β-CN (f 193–209) was detected in bitter S3 cheese, the peptide that accumulated to highest concentration in S3 cheese was α_{S1}-CN (f 1–9) (Figure 5 and Table 4). Because total CEP activity was always several -fold higher in S3 than in S1 or S2 cheeses (see Results), this result was probably a consequence of both group h CEP specificity (Figure 2C) and high total CEP activity. The α_{S1}-CN (f 1–9) peptide has not been linked with bitterness, but the following evidence suggests that α_{S1}-CN (f 1–9) may contribute to that defect. First, peak Q provided good correlation to bitter flavor intensity, and α_{S1}-CN (f 1–9) was the only peptide isolated in the study that eluted in peak Q (Table 3). Second, α_{S1}-CN (f 1–9) has a mean hydrophobicity that is greater than 1400 and a mass that is less than 6000. These properties, according to Ney's Q rule (20), predict this peptide to be bitter. Third, even though α_{S1}-CN (f 1–9) was detected in nearly all cheeses, its concentration was always highest in S3 cheese (Figure 5 and Table 4). This observation is consistent with the hypothesis that bitterness develops when the level of a constituent bitter peptide exceeds its taste threshold (17). Finally, Lee et al. (15), recently found that hydrolysis products of α_{S1}-CN (f 1–23), including α_{S1}-CN (f 1–7) and α_{S1}-CN (f 1–13), contributed to bitterness in Cheddar cheese. Sensory studies are underway in our laboratories to establish the bitter taste threshold for α_{S1}-CN (f 1–9) in cheese and to determine whether it contributes to bitterness in Cheddar cheese.

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